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NEFOPAM ENANTIOMERS: ISOLATION AND ANTINOCICEPTIVE ACTION
[Nefopam-Enantiomere: Isolierung und antinocizeptive Wirkung]

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The analgesic Nefopam (Ajan^{R}, structural formula in the figure) /342* has a chirality center due to the asymmetrically substituted C atom of the benzoxazine ring. It is in commerce as a racemate; the exact action mechanism is still unknown. Since optical isomers of pharmaceuticals often differ in their action[1)] and these differences in action are especially pronounced in analgesics such as methadone[2)], we produced both enantiomers of Nefopam by chromatographic and classic racemate separation and studied them for antinociceptive action. Production, physical data and the pharmacological action of the enantiomers have not been published to date, to our knowledge, although of the Nefopam enantiomers, metabolization[3)] and an x-ray structural analysis[4)] (levorotary Nefopam which was made available[5)] for this purpose by the manufacturer Riker Laboratories is R-configured according to our research) have been described.

Chromatographic racemate separation

Nefopam is largely separated as a base in chromatography on an analytic column of the optically active adsorbent cellulose triacetate with the mobile solvent ethanol. In agreement with previous studies[6)] this separation is promoted by the unsubstituted phenyl residue on the chirality center. On optically active polyamides the racemate is conversely not separated, since hydrogen bridge bonds to the amide groups of the adsorbents are not possible.

For preparative isolation of the two enantiomers, 1.20 and 1.80 g Nefopam base (figure) were chromatographed on 380 g cellulose triacetate. The racemate is almost completely separated on this longer column. From the eluate fraction, 94% (separation test with 1.20 g) and

* Numbers in the margin indicate pagination in the foreign text.

91% (separation tests with 1.80 g) of the respectively supplied racemate were isolated as pure enantiomers. The fractions of (+) and (-) Nefopam with constant specific amounts of rotation from the test with 1.2 g racemate were combined and after distillation were converted into the hydrochlorides. Here 0.57 g (+) and 0.55 g (-) Nefopam hydrochloride (87 and 79% respectively) were obtained in crystalline, analytically pure form. The enantiomer purity of the two preparations is guaranteed by repeated chromatography of a sample as the base on an analytic cellulose triacetate column. Only one UV signal at a time and the pertinent curve of the amounts of rotation with positive and negative recorder deflection were recorded. The admixture of racemate could be clearly detected by deflection of the polarimeter curve in the opposite direction at the start and end of elution. As of many other chiral pharmaceuticals[7,8], thus also of Nefopam, both enantiomers can be quickly and easily isolated by chromatographic racemate separation.

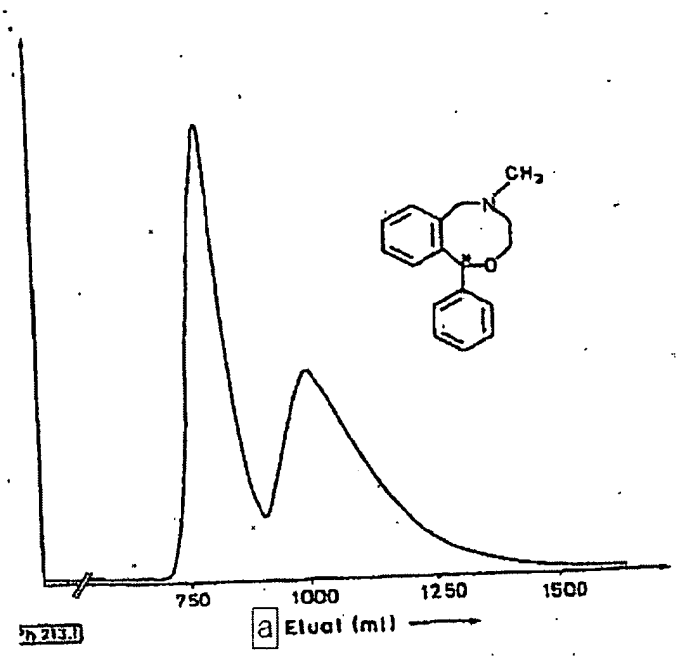

Figure. Chromatography of 1.80 g Nefopam base on 380 g cellulose triacetate, mobile solvent 96 percent ethanol.
------ extinction curve (380 nm)
Key: a) eluate

Separation by way of diastereomer salts

To produce larger amounts of the two enantiomers, crystallization tests of the Nefopam base with equimolar amounts of commercial, optically active acids were done first. Crystalline salts were obtained in 6 batches, the Nefopam enantiomers hardly having been concentrated in the salts with L(-) malic acid, L(+) tartaric acid and L(+) glutaminic acid. Higher amounts of rotation were achieved with 3-bromo(+)-camphor-8-sulfonic acid, ditoluyl- and dibenzoyl-L-tartaric acid. Preparative separation was done with dibenzoyl-tartaric acid which had separated the Nefopam enantiomers best in a preliminary test and which can be obtained from the two enantiomers. After a total of five times fractionated crystallization of the diastereomer salt with

dibenzoyl-L-tartaric acid, the specific amounts of rotation remained constant. The levorotary Nefopam base was liberated from the levorotary dibenzoyl tartrate, distilled in a bulb tube, precipitated as hydrochloride and recrystallized. Here (-) Nefopam hydrochloride was obtained with a melting point of 198-199° (melting point of the racemate 242-255°) in a yield of 20.6%, relative to the (-) enantiomer which was contained in the racemate used. The mother liquors of the first crystallization of the dibenzoyl-L-tartrate contained Nefopam which was concentrated on the (+) enantiomer, and which was recrystallized as the salt of dibenzoyl-D-tartaric acid and was processed accordingly. Then (+) Nefopam hydrochloride of a melting point 198-199° in a yield of 17.6% was obtained. The identity and purity of the two enantiomers are guaranteed by NMR spectra and mass spectra, DC, element analysis and by chromatography on an analytic
cellulose triacetate column. Furthermore, the specific amounts of /344
rotation of (-) and (+) Nefopam are quantitatively the same and agree with the values of the chromatographically separated samples.

Pharmacological testing of Nefopam enantiomers for antinociceptive action

The Nefopam enantiomers and for comparison the racemate[9] were tested for antinociceptive action as hydrochlorides in a hot plate test and in a phenyl quinone writhing test on female NMRI mice. The test substances dissolved in a physiological saline solution were applied intraperitoneally. The hot plate test was conducted 30 minutes after injection of the test substances, the writhing phenomenon was initiated with a dose of 2 mg/kg phenyl quinone (i.p.).

In the hot plate test (Table 1), (+) Nefopam was hardly any more strongly active than the racemate, but significantly more effective than (-) Nefopam (statistical evaluation at 8 mg/kg; $p < 0.025$; at 16 mg/kg: $p < 0.005$). In any case the Nefopam in the hot plate test is less effective[10]. In the writhing test (Table 2) the protective action of the Nefopam enantiomers and of the racemate was studied. The actions of the (+) and (-) enantiomers are highly significantly different in doses of 8 and 32 mg. From the values in Table 2, 50% protection (ED_{50}) at a dose of 3.2 mg/kg (+) Nefopam HCl, 5.5 mg/kg racemate and 8.6 mg/kg (-) Nefopam HCl is computed. (+) Nefopam is thus more strongly effective in antinociceptive terms compared to the (-) enantiomer. The differences in action however are not as strongly pronounced as in chiral analgesics of the morphine and pethidine series[2].

Table 1: Antinociceptive action of (+), (±), and (-) Nefopam hydrochloride in a hot plate test

Dosis (mg/kg) [a]	[b] Dauer der "hot plate"-Latenzzeit (sec) (+)-Nefopam-HCl	(±)-Nefopam-HCl	(−)-Nefopam-HCl
0		(Kontrolle: 3.7 ± 0.3[a])	
4.0	4.4 ± 0.6[b]	[c] 5.1 ± 0.4[b]	[d] nicht bestimmt
8.0	5.1 ± 0.4[d]	4.7 ± 0.4[d]	3.9 ± 0.3[c]
16.0	7.0 ± 0.7[d]	6.8 ± 0.5[d]	4.6 ± 0.2[c]
32.0	9.8 ± 1.2[d]	9.0 ± 0.7[d]	7.4 ± 0.7[c]

Key: a) Dose; col. B) Duration of hot plate latency time; c) control; d) not determined

Legend to Table 1:
Number of animals used per test: a) 14, b) 5, c) 10, d) 15. For female NMRI mice 30 minutes after i.p. injection of the test substances (or physiological saline solution in the control group) the hot plate latency time was determined. The maximum reaction time for each animal was 30 s.

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Table 2: Antinociceptive action of (+), (±), and (-) Nefopam hydrochloride in the writhing test. Number of test animals: for test substances 6 each, for the control group, 24. /345

[a] Dosis (mg/kg)	[b] Durchschnittliche Anzahl der "writhing"-Reaktionen (+)-Nefopam-HCl	(±)-Nefopam-HCl	(−)-Nefopam-HCl
0		(Kontrolle: 29.3 ± 3.6)	
2.0	20.8 ± 8.9	[c] nicht bestimmt	nicht bestimmt
4.0	11.8 ± 4.3	19.5 ± 5.7	[d] 20.3 ± 4.6
8.0	3.5 ± 1.8	9.2 ± 2.7	17.3 ± 2.9
16.0	3.0 ± 2.4	2.2 ± 1.2	6.3 ± 2.8
32.0	0	0	4.7 ± 2.5

Key: a) Dose; b) Average number of writhing reactions; c) control; d) not determined

Legend to Table 2:
Female NMRI mice 30 minutes after i.p. injection of the test substances (or physiological saline solution in the control group) were injected with phenyl quinone (2 mg/kg i.p.). The directly following observation time was 20 minutes for each animal.

Experimental part

Analytic chromatography of Nefopam base on cellulose triacetate

A solution of 10.0 mg Nefopam (5-methyl-1-phenyl-3,4,5,6-tetrahydro-1H-2.5-benzoxazine) as the base in 0.2 ml 96 percent EtOH was chromatographed on a column of 9.0 g microcrystalline cellulose triacetate[6] (column length x diameter = 21.0 x 1.0 cm, dead volume V_o = 12.3 ml) with 96 percent EtOH as the mobile solvent at a slight overpressure (Duramat membrane pump) with a flow velocity of 20 ml/h. With retention volumes of 18.4 and 23.0 ml (+) and (-) Nefopam was largely separated into enantiomers (capacity factors k' = 0.50/0.87,

separation factor α = 1.74). Under the same conditions Nefopam HCl is eluted unseparated closely following the mobile solvent front (k' = 0.38).

Preparative chromatography on cellulose triacetate

The solution of 1.20 g Nefopam base in 8.0 ml EtOH was chromatographed on a column of 380 g microcrystalline cellulose triacetate (column length x diameter = 70.0 x 3.8 cm, dead volume V_o = 470 ml) as described above with a flow velocity of 47 ml/h. With retention volumes of 785 and 1032 ml (k' = 0.67/1.20, α = 1.79, R_S = 1.23), in addition to 130 mg of a mixed fraction, 524 mg (+) base and 500 mg (-) base in enantiomer-pure form were obtained. In the repetition of the chromatography test with 1.80 g Nefopam base in 10.0 ml EtOH (Figure), in addition to 223 mg mixed fraction, 816 mg optically pure (+) base and 694 optically pure (-) base were obtained (k' = 0.63.1.11, = 1.77, R_S = 1.06). The enantiomer-pure (+) and (-) fractions of the first test were combined, evaporated in a vacuum, distilled in a high vacuum in a bulb tube and precipitated from an ether. solution with ether. HCl as hydrochlorides. From the base which is dextrorotary in EtOH, 573 mg (87%, relative to the dextrorotary enantiomer in the racemate used) (+) Nefopam HCl of melting point 195-197° were obtained, $[\alpha]^{22}_{365}$ = 504.4, $[\alpha]^{22}_{D}$ = 121.1 (c = 0.27 in DMSO), from the levorotary fraction which was eluted later 545 mg (79.4%) (-) Nefopam HCl of melting point 194-196° were obtained, $[\alpha]^{22}_{365}$ = 509.1, $[\alpha]^{22}_{D}$ = 123.0 (c = 0.17 in DMSO), samples of roughly 10 mg at a time were chromatographed as bases on the analytic column. In the recorder

diagram only one UV peak at a time and one polarimeter deflection to negative and positive amounts of rotation were recorded.

Preparative separation by way of diastereomer salts /346

Solutions of 0.50 g Nefopam base at a time were mixed with ethanolic solutions of equimolar amounts of the following acids: (-) abietinic acid, N-acetyl-L-leucine, L(-) malic acid, L(+) ascorbic acid, 3-bromo-(+)camphor-8-sulfonic acid, (+) camphor acid, (+) camphor-10-sulfonic acid, (+) 2-chloro-tartranilic acid, (+) 4-chloro-tartranilic acid, (-) di-0,0'-benzoyl-L-tartaric acid monohydrate, (-) di-0,0'-toluoyl-L-tartaric acid monohydrate, D-glucuronic acid, L(+) glutaminic acid, L(+) mandelic acid, L(+) lactic acid, N-[S(-)-(1-phenylethyl)]-phthalic acid monoamide, L(+) tartaric acid. After standing for several days, from the batches with malic acid, glutaminic acid and tartaric acid, salts are obtained which after separation of the acid and measurement of the amounts of rotation of the base fraction indicate almost no separation of the Nefopam. Conversely, the salts of the 3-bromocamphor-8-sulfonic acid, and toluoyl tartaric acid and benzoyl tartaric acid yielded roughly 30 percent Nefopam concentration. In the main test, the solution of the Nefopam base which was liberated from 58.0 g Nefopam HCl (0.20 mole) in 140 ml DMSO with stirring was dripped into an ice-cold solution of 75.3 g (0.20 mole) (-) di-0,0'-benzoyl-L-tartaric acid monohydrate in a mixture of 100 ml DMSO and 360 ml EtOH. After several days, 79.3 g of colorless crystals (optical purity of Nefopam 75%) on monotartrate has settled, melting point 130° (decomp).

$C_{17}H_{20}NO \cdot C_{18}H_{13}O_8$ (611.7) Calculated. C 68.73 H 5.44 N 2.29; Found: C 68.17 H 5.49 N 2.27.

By 5 recrystallizations from DMSO/H_2O the optical purity was raised to roughly 89; 93; 95 and > 95%. To do this, the salt was dissolved in DMSO and the warm solution was mixed with warm water until clouding began. After the last recrystallization, there were still 15.1 g dibenzoyl tartrate, from which the (-) Nefopam base was liberated with NaOH/ether, distilled in a high vacuum at 145° in a ball tube and was precipitated with etheric HCl as the hydrochloride from ether. After recrystallization twice from EtOH/H_2O (1:1), 6.0 g (20.6% relative to the levorotary Nefopam in the racemate used) (-) Nefopam HCl of melting point 198-199° were obtained, $[\alpha]^{22}_{365}$ = 520.9, $[\alpha]^{22}_{D}$ = 125.9 (c = 0.16 in DMSO).

The mother liquor of the first, above described crystallization of 0.2 mole racem. Nefopam was dripped into 1.5 l ice water. The precipitated salt concentrated on (+) Nefopam was decomposed, the dextrorotary Nefopam base (15.3 g) with 37.7 g (+) dibenzoyl tartaric acid was caused to crystallize and was recrystallized twice from DMSO/H_2O, after which an optical purity of > 95% was achieved. Decomposition into the base, distillation and crystallization twice as the hydrochloride yielded 5.1 g (17.6% relative to the dextrorotary form which is contained in 68 g racemate) (+) Nefopam HCl of melting point 198-199° (decomp.) were obtained, $[\alpha]^{22}_{365}$ = 519.9, $[\alpha]^{22}_{D}$ = 125.1 (c = 0.94 in DMSO).

$C_{17}H_{20}NO \cdot C_{18}H_{13}O_8$ (289.8) Calculated. N 4.83; found for (-) Nefopam HCl N 4.68 for (+) Nefopam HCl N 4.57. Both enantiomers chromatographed as bases on the analytic cellose triacetate column showed only one UV peak and a peak of amounts of rotation. Admixture of 2.5% of the respectively other enantiomer could be clearly detected in the diagram

of amounts of rotation of the starting and ending fractions by recorder deflection in opposite directions. The enantiomers are configuration-stable under physiological conditions: an aqueous ethanolic solution of pH 7.4 after 24 hours at 37° did not show any decrease of the amount of rotation.

Pharmacological test of Nefopam enantiomers for antinociceptive action

The hydrochlorides of the Nefopam enantiomers and of the racemate, dissolved with slight heating in physiological saline solution, were applied intraperitoneally to female NMRI mice with a weight of roughly 22-24 g. The animals were ordered from Charles River Wiga, D-8741 Sulzfeld 1. The injection volume was 10 ml/kg body weight. In the slightly modified hot plate method given by Woolfe and MacDonald[11] the mice were placed for up to 30 s on a copper plate (diameter 20 cm, with the edge 8 cm high and likewise temperature-controlled) which is kept constantly at this temperature and which is heated by hot water to 56 ± 0.2°. The animals could not jump off the plate due to a plexiglass cylinder 40 cm tall which had been placed on this edge. The measurement time was the time from placing the animals on the plate to a specific /347 stimulus response, leaking of the front or (very isolated) rear paws. Time measurement in s ± 0.1 s.

The writhing test was done with slight modifications after Hendershot and Forsaith[12]. A dose of 2 mg/kg 2-phenyl-1,4-benzoquinone was intraperitoneally injected into the mice 30 minutes after intraperitoneal injection of the test substances or physiological saline solution. Directly afterwards the animals were placed individually in round plastic cages with wire covering (15 cm diameter and 5.5 cm tall); in the following 20 minutes the number of writhing

reactions was recorded using a counting mechanism. Only complete extension of one or both back legs was evaluated. Reactions which consisted solely of a contraction of the abdominal musculature, drawing in of the flanks and torsion of the body were rated as incomplete "writhing" and were not recorded. The phenyl quinone solution was freshly produced on each test day: 10 mg phenyl quinone (Eastman Organic Chemicals) were dissolved in an opaque vessel with 2.5 ml absol. EtOH at 40° and then demineralized water of 40° was added to make 50 ml. This solution was stored at roughly 35° in a heat cabinet. The injection volume of this solution was 10 ml/kg body weight. The control groups were treated accordingly with physiological saline solution. The animals were used repeatedly after an at least 5-day test pause. Statistical evaluation of the two test series was done with the Mann-Whitney-Wilcoxon test.

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